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THE EFFECTS OF FOUR DRUGS ON CHINESE HAMSTER OVARY CELLS

by

© HELENA SROKA

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND

RESEARCH IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

DEPARTMENT OF GENETICS

EDMONTON, ALBERTA

FALL, 1971

ABSTRACT

The effects of four drugs: chlorpromazine, dimethyl sulfoxide, p-brombenzylisothiocyanate and p-bromphenylisothiocyanate on Chinese Hamster ovary cells *in vitro* were studied. A dose-survival curve was derived for each agent and its influence on mitotic index was measured. Production of chromosomal aberrations after exposure to these drugs was studied under standardized conditions. Chlorpromazine and p-bromphenylisothiocyanate did not induce aberrations under the experimental conditions employed. Treatment with 3% dimethyl sulfoxide resulted in the production of chromosomal aberrations in 5-14% of the mitotic cells (harvested 0-12 hr after treatment). With prolonged culture time after treatment, chromosomal defects were found to be diminished. After exposure of cells to p-brombenzylisothiocyanate, there was a marked increase in the number of polyploid cells.

ACKNOWLEDGEMENTS

I should like to thank Dr. J. Weijer for his support throughout this study and for his criticisms in the preparation of this thesis. I am indebted to Mr. J. Miller for his help in proof-reading and typing of the manuscript. My thanks are extended to Mr. D. Walker for preparing the Plates in this thesis. This work was supported by a National Research Council of Canada grant to Dr. J. Weijer.

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INTRODUCTION

Exogenous agents such as radiations, several viruses and a variety of chemicals cause chromosomal damage. Because of their eventual genetic effects, agents causing chromosomal damage are potentially dangerous and therefore their study is relevant.

Refinement of tissue culture techniques permits the study of specific treatments of mammalian cells. In this investigation, Chinese Hamster ovary (CHO) cells *in vitro* were used. Interest was focused on chemicals with pharmacological importance:

(1.) chlorpromazine, a tranquilizing drug widely used in the therapy of neurological disorders;

(2.) dimethyl sulfoxide, a drug used as a penetrating carrier and local analgesic in medicine and as an antifreeze agent in animal tissue culture; and

(3 & 4.) p-brombenzylisothiocyanate and p-bromphenyl-isothiocyanate, known for their antibacterial, antiviral and antihelminthic properties.

The effects of these agents on the growth of CHO cells and production of chromosomal aberrations were studied.

LITERATURE REVIEW

Since the first demonstration of chromosomal breaks induced by chemicals (Oehlkers, 1943), research dealing with the effects of chemicals on chromosomes has become very extensive. A variety of chemicals have been demonstrated to cause chromosomal damage; among them, nucleic acid precursors and related substances, alkylating agents, nitrosocompounds, antibiotics, and a heterogeneous group of other chemicals (see reviews of Kihlman, 1966, and of Barthelmess, 1970). Technical improvements such as the use of hypotonic shock (Hsu, 1952), use of colchicine for the accumulation of cells in metaphase (Tjio and Levan, 1956), the culture of phytohaemagglutinin-stimulated lymphocytes (Moorhead, et al., 1960; Nowell, 1960), etc., further stimulated research in this field. The test system most frequently employed today involves the culture of cells in vitro. Experiments in vitro are relatively simple in that they permit detailed analysis of various aspects of a system, viz. cell cycle determination, occurrence of specific biochemical events during the cell cycle, clonal growth of isolated cells, etc. Chinese Hamster ovary cells and cells derived from marsupials are most suitable for in vitro cytogenetic studies because the karyotype has a small number of chromosomes. However, conditions in vivo cannot be precisely imitated and therefore experiments in vitro have several limitations.

Chemicals induce different types of chromosomal aberrations. Some agents act specifically, whereas others cause a broad spectrum of chromosomal defects. Activity of some chemicals is restricted to

certain chromosomes or sites on chromosomes (Revell, 1953; Kihlman, 1961), while others exert their effects randomly. Investigators differ in their criteria for scoring chromosomal aberrations. For example, achromatic lesions in the chromatids observed without dislocation of segments are interpreted by some authors as chromatid or subchromatid breaks (Marquard, 1950; Freed and Schatz, 1969), or as a specific defect (Gebhard, 1969), whereas others do not consider this to be the case (Revell, 1959).

Although there has been a great deal of investigation in the field of chemically induced chromosomal damage, very little is known of the mechanism(s) by which chromosomal anomalies are mediated. Several mechanisms have been postulated, including inhibition of the synthesis of DNA and DNA precursors, degradation and denaturation of DNA, production of abnormal DNA, removal of DNA-bound metals (reviewed by Kihlman, 1966). Freed and Schatz (1969) proposed that interruption of DNA replication and consequent chromosome aberrations are the result of the inhibition of protein synthesis. Wolff and Luippold (1955) demonstrated that repair processes of chromosomal damage were inhibited by inhibitors of cellular respiration and of ATP formation. Repair processes also depend on protein synthesis (Wolff, 1960). Allison and Paton (1965) suggested that chromosomal damage may be induced by the release of lysosomal enzymes.

A brief review of recent work done on four drugs used in this study is presented here. Cohen, Hirschhorn and Frosch (1967) reported an elevated frequency of chromatid and chromosomal breaks in leucocytes cultured from three patients who had been treated with chlorpromazine (CPZ). Jenkins (1970) confirmed this in peripheral blood from

individuals under treatment with phenothiazines (chlorpromazine and trifluoperazine). Green, Palmer and Legator (1970) demonstrated chromosomal rearrangements as well as chromatid breaks induced by triflupromazine in rat-kangaroo cells in vitro.

Later studies of Cohen, Hirschhorn and Frosch (1969) carried out both in vivo and in vitro on human leucocytes with a variety of phenothiazines (chlorpromazine, thioridazine hydrochloride and fluphenazine hydrochloride) did not demonstrate chromosomal aberrations. In addition, the results of Kamada (1971) did not show chromosomal damage to human leucocytes exposed to CPZ in vitro.

Westring, et al. (1964) and Bloom and Tjio (1964) agreed that CPZ causes a decrease in mitotic activity and blast cell formation. These effects could be attributed to the inhibition of enzymes (Marks, Roesky and Carver, 1961; Hülsmann, Fabius and De Ruiter, 1964), or to effects on the cell membrane. CPZ inhibits cell permeability of human erythrocytes (Freeman and Spirtes, 1963) and of rat liver mitochondria (Spirtes and Guth, 1963). CPZ at low concentrations stabilizes lysosomes (Guth, et al., 1963), whereas, at high concentrations, it ruptures them (Jacques, Ennis and de Duve, 1964). CPZ was found to inhibit the incorporation of tritiated thymidine into the DNA of bone marrow cells (Pisciotta and Kaldahl, 1962).

It has been demonstrated that dimethyl sulfoxide (DMSO) affects a number of biological processes. Stenchever, Hopkins and Sipes (1967) found that a 1% solution of DMSO causes a lag in the generation time of human fibroblast cultures in vitro, but they observed no chromosomal damage. Chang and Simon (1968) reported that DMSO retards the growth of HeLa cells, L-cells, E. coli, Mengo virus, and

bacteriophage T_4. The same drug was found to inhibit protein synthesis in bone marrow cells *in vitro* (Ashwood-Smith, 1967) as well as to inhibit amino acid transport (Hagenann and Evans, 1969). Results of the experiments with *in vitro* systems showed that DMSO affects the activities of a variety of enzymes (Rammler, 1967; Chang, 1968). Ferm (1966) reported teratogenic effects of DMSO in golden hamsters, and Caujolle, *et al.* (1967) observed similar effects in chickens and rats. Archer (1967) reported dilation of the endoplasmic reticulum and diminution of ribosomes in the fibroblasts of rats after treatment with DMSO. Misch and Misch (1969) demonstrated reversible activation of rat liver ribosomes by DMSO.

Many natural and synthetic isothiocyanates (ITC's) were found to possess remarkable biological activities. The first noted was their antibacterial effect (Milton and Foter, 1940; Haag, 1941). Later, their antihelminthic properties (Sprau, 1965; Gee, Fink and Beaver, 1966), antiprotozoal (Balanova, 1968) and antifungal properties (Drobnica, *et al.*, 1967) were demonstrated. A cancerostatic effect upon Ehrlich Ascites cells (Nemec, et al., 1958) upon the 3,4-benzpyrene-induced carcinoma of mice (Balan and Drobnica, 1961) and upon Yoshida sarcoma (Miko and Ujhazy, 1968) was reported. Horakova, *et al.* measured the cytotoxicity of ITC on HeLa cells. The mechanism of action of ITC and the relation between chemical structure and its biological effects was intensively studied. ITC is believed to inhibit the glycolytic pathway (Drobnica, 1961). The target of some ITC's in the glycolytic pathway of yeast, Ehrlich Ascites carcinoma cells and HeLa cells is the inhibition of glyceraldehyde-3-phosphate dehydrogenase. Some ITC's also inhibit

RNA and DNA synthesis (Drobnica, 1967). The effects of the ITC's on chromosomes have not, as yet, been studied.

MATERIALS AND METHODS

A. Cell Culture

Chinese Hamster ovary cells (*Cricetulus griseus*), strain CCL-61, were obtained from the American Type Culture Collection Cell Repository, Rockville, Md. A cell subline with 21 chromosomes was derived by repeated cloning and was used in all experiments. To ensure karyotypic homogeneity of the subline, cloning was performed every second month. The cells were propagated routinely as monolayers in 75 cm^2 and 25 cm^2 plastic flasks (Falcon Plastics Division, Becton, Dickinson, and Co.). McCoy's 5a (modified) culture medium supplemented with 10% heat-inactivated calf serum, 5% heat-inactivated fetal calf serum, 50,000 IU/litre penicillin, 50,000 mg/litre each of streptomycin and neomycin, with 2.2 g/litre of sodium bicarbonate was used for cell maintenance and experiments. This medium was prepared from commercial components obtained from Grand Island Biological Company, Grand Island, N.Y. (GIBCO). The cells were incubated at 37°C in a humid atmosphere of air supplemented with 5% CO_2. A 0.05% solution of trypsin (Schwarz Bioresearch Inc., N.Y.) in Hank's balanced salts solution was used (37°C for 1 min) to detach cells from the flasks.

For the purpose of taking photomicrographs of the morphology of CHO cells (comparison of CPZ-treated and control cells), the cells were grown on glass coverslips (10.5 x 50 mm, Bellco Glass) in Leighton tubes containing 2 ml of culture medium. Cells were in contact with the drug for 17 hr (controls were incubated for the same period) and then fixed on the coverslips in methanol for 2 min. The preparation was stained using the Harris Haematoxylin-Eosin

method (Merchant, et al., 1960) and mounted with Euparal on microscope slides.

B. Isolation of Clones

Clones were isolated after the procedure of Ham and Puck (1962), as modified by Giblak (personal communication). Single cells were plated at low dilution. After incubation for 6-8 days, macroscopic clones were located and their position marked. The top of the flask was perforated with a cork-borer and colonies were released from the plastic with trypsin applied through a blood collecting pipette. At this time, the cells were aspirated into the pipette with a tuberculin syringe and transferred to fresh medium.

C. Chemicals

The four drugs of interest were obtained from the following sources: chlorpromazine hydrochloride (CPZ), Paul Maney Laboratory, Canada, Ltd.; dimethyl sulfoxide (DMSO) , Fisher Scientific Co.; p-brombenzylisothiocyanate (PBBI) and p-bromphenylisothiocyanate (PBFI) were obtained through the courtesy of K. Horakova and were prepared at the Slovak Technical Institute. Chlorpromazine was dissolved in DMSO. Solutions of drugs were stored in the frozen state.

D. Determination of Generation Time

Approximately 2.5×10^5 cells were incubated in each of 15 of the smaller flasks for 45 hr. Cell number was estimated with a Hawksley Cristalite Hemacytometer, Sussex, England. The medium was then decanted and replaced with fresh medium containing 0.08 µg/ml of colcemid. Cells were harvested every hour by trypsinization. Both trypsinized cells and those in the culture medium were collected by centrifugation for 5 min at 850 rpm in a clinical centrifuge.

Following fixation in acetic acid-ethanol solution for 2 hr, a few drops of the cell suspension were placed on microscope slides and allowed to dry at room temperature. Slides were stained with Feulgen stain. One thousand cells per slide were scored as interphase or metaphase.

E. Construction of Dose-Survival Curves

Five hundred cells were inoculated into 75 cm^2 plastic flasks. After 3 hours' incubation, the chemical of interest was added (at least 2 replicate flasks) in specified concentrations, and incubation continued for 17 hr. The cells were then washed twice with Hank's balanced salts solution and fresh medium was added. After 6-7 days' incubation, macroscopic clones were stained with crystal violet-citric acid stain and counted. Plating efficiency in all experiments was approximately 90%.

F. Observation of Chromosomes

Drugs were tested under constant conditions for the induction of chromosomal aberrations. Approximately 10^6 cells were inoculated into each of a series of 75 cm^2 flasks and incubated for 4-5 hr. The medium was then decanted and replaced with medium containing a specific drug (a concentration which gave 20-40% survival). After an incubation period of 17 hr, the cells were washed twice with Hank's balanced salts solution and this was defined as t_0. To the series of flasks, colcemid was added at different times (usually 4, 8, 12, 16, 24, 36 and 48 hr) after washing the cells. A series of control flasks was set up under the same experimental conditions.

Macroscopic colonies resulting from treatment with specific drug concentrations were also examined for chromosomal aberrations.

Clones were trypsinized, transferred to 25 cm^2 flasks, and grown to confluency.

In every experiment mitotic cells were accumulated during 3 hr after the addition of colcemid in Hank's balanced salts solution (GIBCO) at a final concentration of 0.08 μg/ml. Mitotic cells were recovered from the flasks by gentle shaking and decanted with the medium. This suspension was centrifuged in a clinical centrifuge at 850 rpm for 7 min. The cell pellet was resuspended in hypotonic solution (0.95% sodium citrate) for 15 min at 37°C. After centrifugation at 850 rpm for 10 min, the cells were fixed with acetic acid-ethanol solution (1:3) for a minimum of 2 hr at 4°C. Within 24 hr post-fixation, cells were resuspended in fresh fixative (acetic acid-methanol solution, 1:3). A few drops of cell suspension were placed on a slide previously dipped in glacial acetic acid, air-dried at room temperature and stained with 1.5% aceto-orcein.

More than 100 mitoses were examined in each particualr experiment and scored as, normal karyotype with 21 chromosomes (typical metaphase in Plates 3 and 4), polyploid cells (Plates 5 and 6), and cells with other than 21 chromosomes. Structural aberrations including breaks of the chromatid and isochromatid types, polycentric chromosomes, rings, acentric fragments, and exchange figures were counted (see Plates 7-9). Due to ambiguity in the literature, chromatid gaps were not included in the study. Cells with several exchanges were counted only once.

G. Measurement of Mitotic Index

The mitotic index (mean number of mitotic cells per 1000 cells counted) was determined by counting at least 1000 cells. Various

concentrations of a tested drug were added to cultures of actively growing cells in 25 cm^2 flasks. After 17 hr, the cells were harvested by trypsinization and decanted with the medium. The cells collected by centrifugation were fixed with acetic acid-ethanol solution for 2 hr, and slides were prepared as described above.

To determine whether the mitotic index was significantly changed by treatment with drugs Chi-square tests were applied. The level of significance was set at 0.05.

H. Photomicrography

Observations and photomicrographs were made with a Leitz Orthoplan microscope with a 90x objective and 10x ocular. Photomicrographs were taken with a Leitz Orthomat 35 mm camera and a green filter was used. High contrast copy film was developed in Kodak D 19 developer for 6 min at 20°C.

RESULTS

A. Determination of Generation Time

The accumulation of metaphase cells in an asynchronous population blocked by colcemid corresponds to Equation (4) of Puck and Steffen (1963):

$$\log (1 + N(M)) = \frac{0.301\ t}{T_C} ,$$

for $t \geq T(M)$, where $N(M)$ is the fraction of cells in metaphase, T_C is the total generation time, $T(M)$ is the duration of mitosis and t is time elapsed since the addition of colcemid. A plot of $\log (1 + N(M))$ vs t gives a straight line with slope $\frac{0.301}{T_C}$ (for $t>1$) from which T_C can be determined.

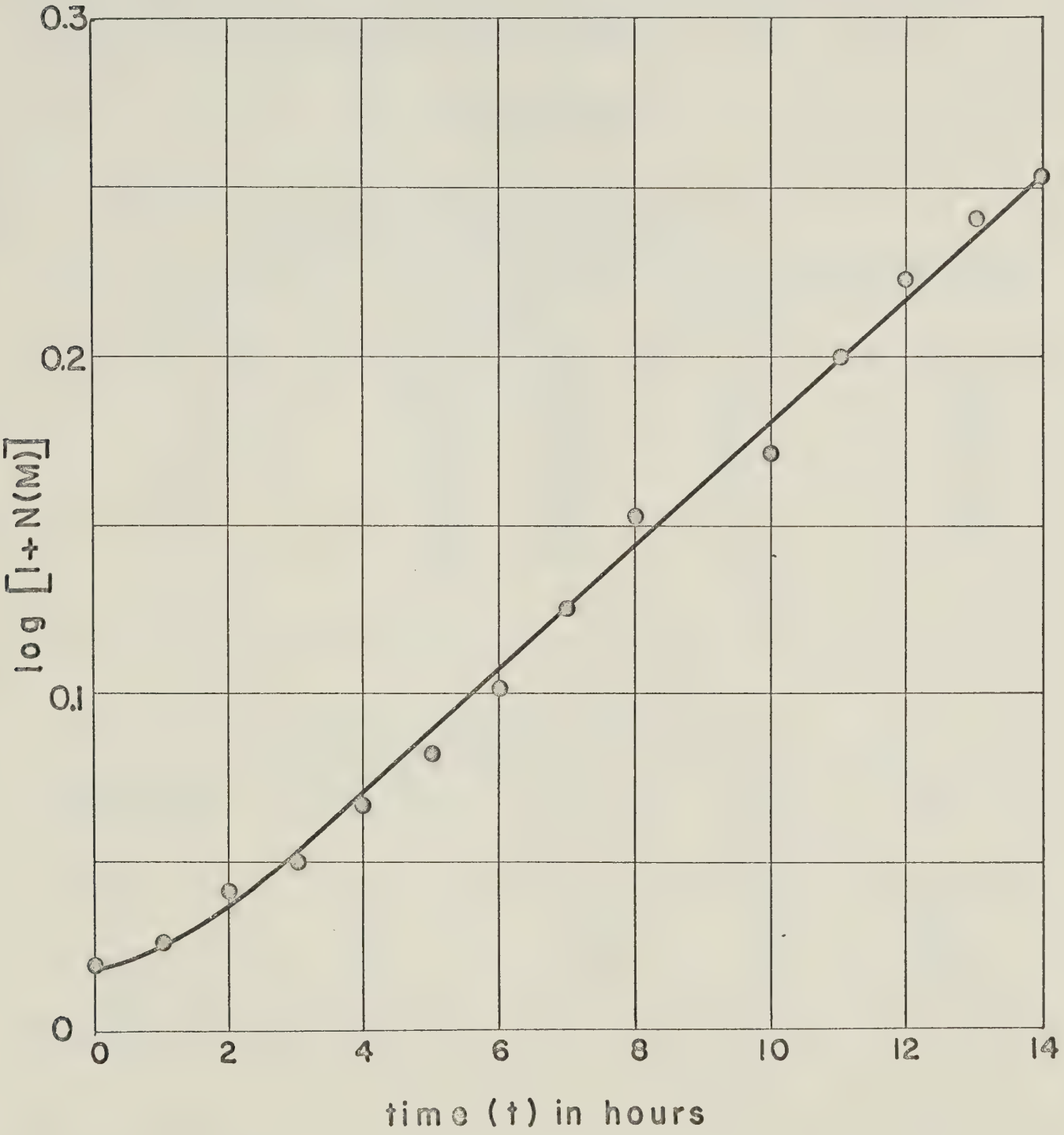
From the data, the slope = 0.0181.

Therefore, $T_C = \frac{0.301}{0.0181}$, from which T_C is found to be 16.63 hr.

Table I. Accumulation of Metphase Cells in an Asynchronous Population Subjected to Colcemid

Time (hr)	Mitoses	Interphases	Total	(M)	log (1 + N(M))
0	42	980	1022	0.042	0.0170
1	58	952	1010	0.057	0.0253
2	108	964	1172	0.097	0.0414
3	148	1102	1250	0.118	0.0492
4	194	979	1173	0.165	0.0645
5	256	942	1198	0.214	0.0828
6	310	902	1212	0.255	0.1004
7	423	807	1230	0.343	0.1271
8	528	723	1251	0.422	0.1523
9	558	690	1248	0.447	0.1614
10	620	679	1299	0.479	0.1703
11	810	524	1334	0.607	0.2068
12	922	430	1352	0.681	0.2253
13	1001	290	1291	0.770	0.2480
14	1050	266	1316	0.797	0.2553

Fig. I. Accumulation of Metaphase Cells in an Asynchronous Population Subjected to Colcemid


0.3
0.2
0.1
0
log [1+ N(M)]
0
2
4
6
8
10
12
14
time (t) in hours

B. The Effects of Four Pharmacological Agents on Survival, Mitotic Index and Chromosomes

The following tables and figures summarize the effects of four pharmacological agents on survival, mitotic activity and chromosomes of asynchronously growing CHO cells *in vitro*.

1. Chlorpromazine

Table II. Survival of CHO Cells After Exposure to Different Concentrations of CPZ

Concentration of CPZ (μg/ml)	Number of Clones Replicate 1	Number of Clones Replicate 2	Average Number of Clones	% Survival
0	473	457	465.0	100.0
6	422	418	420.0	90.3
12	383	358	370.5	79.7
15	295	358	326.5	70.2
18	207	213	210.0	45.2
21	79	82	80.5	17.3
24	24	24	24.0	5.2
27	7	9	8.0	1.7
30	0	0	0.0	0.0

Table III. Influence of Different Concentrations of CPZ on the Mitotic Index

Concentration of CPZ (μg/ml)	Number of Mitotic Cells	Number of Interphase Cells	Total Number of Cells Counted	Mitotic Index
0	35	1017	1052	33.3
3	17	1062	1079	15.7*
15	10	1004	1014	9.8*
30	0	1000	1000	0.0*

*Significant at the 0.05 level.

Fig. II. Dose-Survival Curve of CHO Cells Exposed to Different Concentrations of CPZ

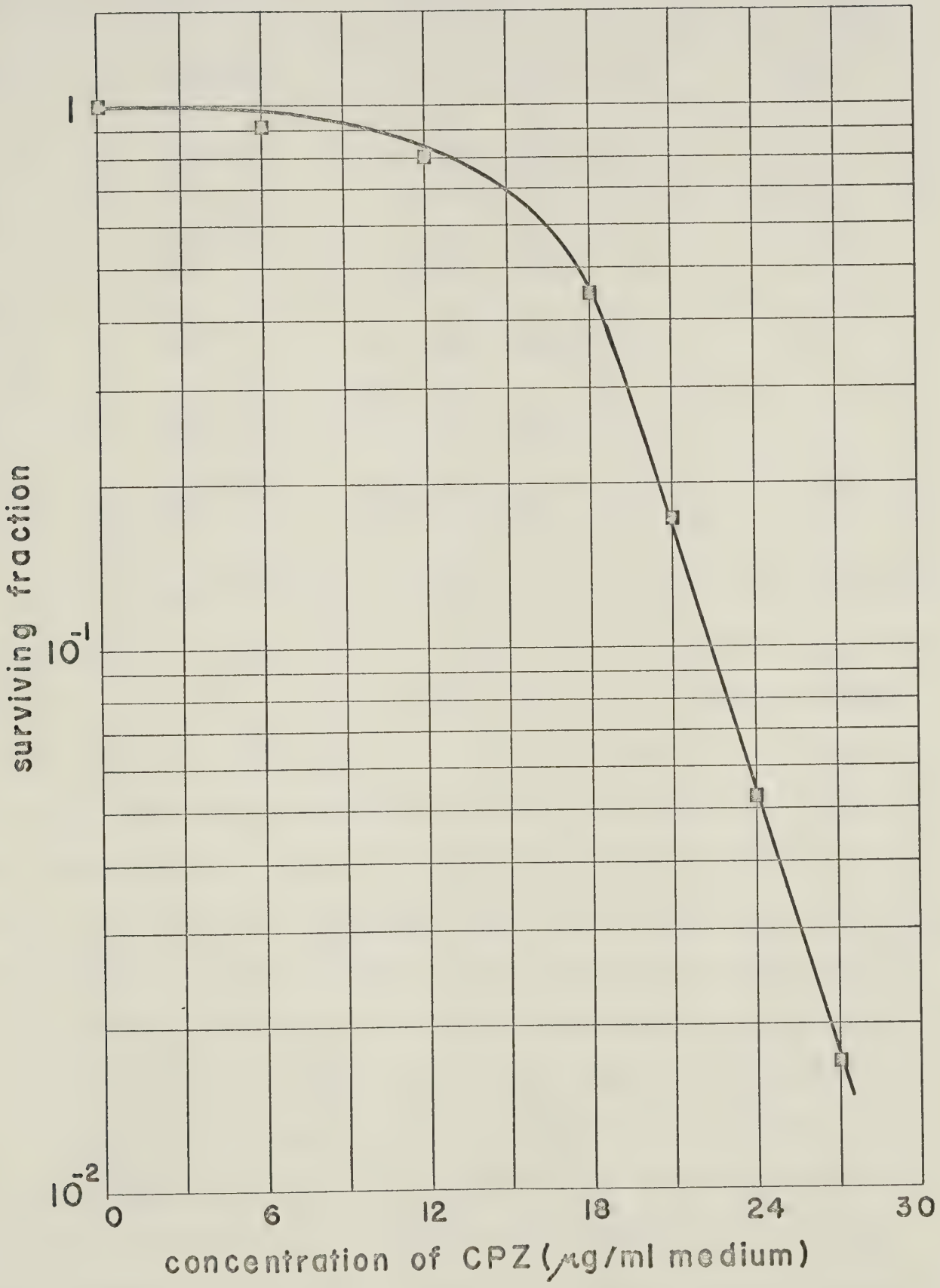

surviving fraction
1
10^{-1}
10^{-2}
0
6
12
18
24
30
concentration of CPZ (μg/ml medium)

Table IV. Effect of CPZ (20 μg/ml) on Chromosomes of CHO Cells at Various Intervals After Treatment

Time (hr)	Treatment	% Metaphases With Chromosome Number					
		19	20	21	22	23	polyploid
t_0	Control	-	1.9	91.3	2.9	-	3.8
	CPZ	-	5.0	89.0	-	1.0	5.0
4	Control	-	3.9	89.1	1.9	-	4.9
	CPZ	-	2.9	89.1	2.9	0.9	3.9
8	Control	-	4.8	95.1	1.0	-	1.9
	CPZ	-	6.9	90.1	-	-	3.9
12	Control	-	2.8	92.4	2.8	-	1.9
	CPZ	-	0.9	94.3	0.9	-	3.7
24	Control	1.0	1.0	91.1	2.9	-	1.0
	CPZ	-	0.9	96.2	2.8	-	-
48	Control	1.0	2.0	93.1	3.0	-	1.0
	CPZ	1.0	1.9	94.2	2.9	-	-

The relationship between the concentration of CPZ and the fraction of the CHO cell population which retains its cloning ability is demonstrated in Table II. As can be seen in Fig. II., the dose-survival curve continues exponentially after an initial shoulder. At concentrations higher than 30 μg CPZ/ml, no clones were observed. D_{10} was defined as that dose required to reduce the surviving fraction by a value of 10^{-1}, and was determined from the curve to be equal to 22 μg CPZ/ml. Different concentrations of CPZ were also examined for their effects on the mitotic activity of asynchronously growing CHO cells (Table III.). It was found that even very low concentrations of CPZ (3 μg CPZ/ml) significantly decreased the mitotic index. In every experiment, the cells were exposed to the drug for about one generation time (17 hr). In studies of the effect of CPZ on chromosomes of CHO cells, a concentration of 20 μg CPZ/ml was used because of experimental convenience. This dose permits 22% cell survival.

Observations of chromosomes at various intervals after CPZ treatment (Table IV.) did not show noticeable differences when compared with controls. CPZ was not found to induce chromosomal aberrations, aneuploidy or polyploidy under the conditions employed. Cells treated with concentrations of CPZ higher than 21 μ g/ml appeared to be changed in morphology. They became smaller and more round in shape when compared with untreated fibroblasts (See Plates I. and II.).

2. Dimethyl Sulfoxide

Table V. Survival of CHO Cells Exposed to Different Concentrations of Dimethyl Sulfoxide

Concentration (ml DMSO/ml medium)	Number of Clones Replicate 1	Replicate 2	Average Number of Clones	% Survival
0.000	468	454	461.0	100.0
0.010	417	385	401.0	86.9
0.020	320	288	304.0	65.9
0.030	126	163	144.5	31.3
0.035	37	28	32.5	7.0
0.040	10	4	7.0	1.5
0.050	0	0	0.0	0.0

Table VI. Influence of Different Concentrations of DMSO on the Mitotic Index

Concentration (ml DMSO/ml medium)	Number of Mitotic Cells	Number of Interphase Cells	Total Number of Cells Counted	Mitotic Index
0.00	48	1310	1358	35.3
0.01	31	1122	1153	26.8
0.02	33	1164	1197	27.5
0.03	16	1004	1020	16.0*
0.04	0	1000	1000	0.0*

*Significant at the 0.05 level.

Fig. III. Dose-Survival Curve of CHO Cells Exposed to Different Concentrations of DMSO

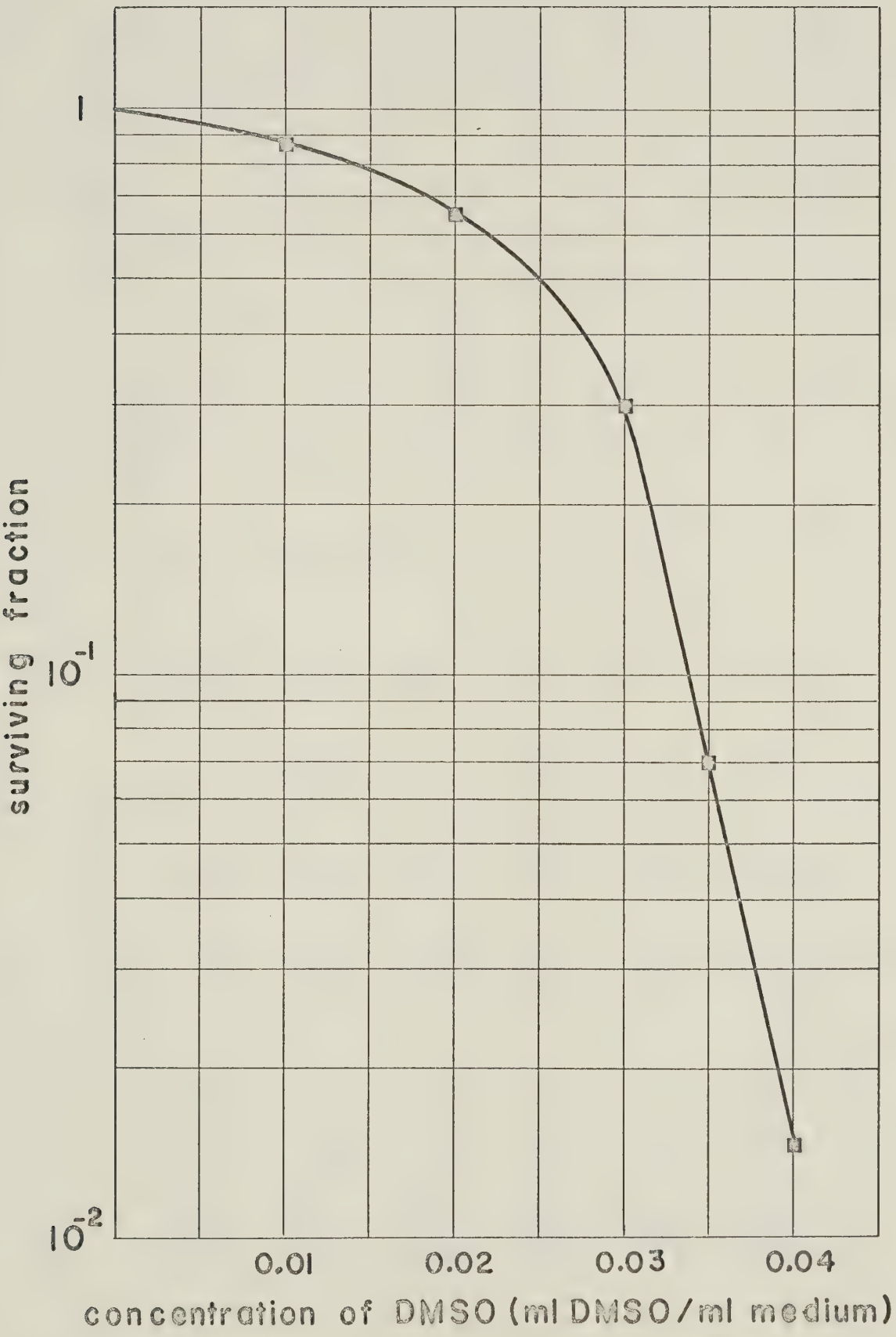

1
10^{-1}
10^{-2}
surviving fraction
0.01
0.02
0.03
0.04
concentration of DMSO (ml DMSO/ml medium)

Table VII. Effect of DMSO (0.03 ml DMSO and 0.01 ml DMSO/ ml of Medium) on Chromosomes of CHO Cells at Various Intervals After Treatment

Time (hr)	Treatment	% Metaphases With Chromosome Number					% Metaphases With Structural Aberrations		
		19	20	21	22	Poly-ploid	Chromatid Exchanges	Dicentrics	Breaks
t_0	Control	-	-	97.0	1.0	2.0	-	-	-
	DMSO (0.03)	-	3.9	77.7	0.9	3.9	7.7	2.0	4.0
	DMSO (0.01)	-	8.0	87.0	1.0	3.0	-	-	-
4	Control	-	4.0	89.1	2.0	4.9	-	-	-
	DMSO (0.03)	-	6.4	85.2	2.5	4.4	6.9	-	1.5
	DMSO (0.01)	-	1.9	92.4	1.9	3.8	-	-	-
8	Control	-	4.9	92.2	1.0	1.9	-	1.0	-
	DMSO (0.03)	-	6.3	81.0	2.7	3.6	5.4	-	0.9
	DMSO (0.01)	-	1.9	96.1	-	1.9	-	-	-
12	Control	-	2.8	92.4	2.8	2.0	-	-	-
	DMSO (0.03)	-	2.6	88.5	0.9	3.5	2.6	-	1.8
	DMSO (0.01)	-	2.0	96.0	1.0	1.0	-	-	-
24	Control	1.0	1.0	91.1	5.9	1.0	-	-	-
	DMSO (0.03)	-	1.9	93.4	2.8	1.9	-	-	-
	DMSO (0.01)	-	1.9	97.1	-	1.0	-	-	-
48	Control	1.0	2.0	93.1	2.9	1.0	-	-	-
	DMSO (0.03)	2.0	5.0	89.0	2.0	1.0	1.0	-	-
	DMSO (0.01)	-	2.9	96.1	1.0	-	-	-	-

Table VIII. Observations of Chromosomes of Surviving Clones After Exposure to DMSO (0.03 and 0.01 ml DMSO/ ml of medium)

Treatment	% Metaphases With Chromosome Number				Dicentrics
	20	21	22	Polyploid	
Control	1.9	97.1	-	1.0	-
DMSO (0.03)	1.0	98.0	-	-	1.0
DMSO (0.01)	1.0	98.0	1.0	-	-

The dose- survival curve for CHO cells exposed to DMSO is presented in Table V. and Fig. III. D_{10} as determined from this curve amounts to 0.034 ml DMSO/ ml medium. The concentration used (0.03 ml DMSO/ml) allowed approximately 30% survival and this concentration was used in studies of the effects of DMSO on chromosomes. Observations of chromosomes after treatment with this concentration of DMSO are summarized in Table VII. An increase in the number of aberrations, especially of exchange figures, was noted. Twenty four hours after washing of cells no aberrations were observed. Because DMSO was used in futher experiments as a solvent (in low concentrations) for isothiocyanates, it was necessary to investigate the effect of lower concentrations of DMSO on chromosomes. Even at a concentration of 0.01 ml DMSO/ml medium (maximal solvent concentration) no aberrations or changes in chromosome number were observed (Table VII.). Similar results were obtained in chromosome studies of cell clones which survived DMSO treatment (Table VIII.) Doses of 0.03 ml DMSO/ml medium and higher were found to reduce the mitotic index significantly, whereas a concentration of 0.01 ml DMSO/ml did not significantly influence this parameter (Table VI.).

3. p-brombenzylisothiocyanate

Table IX. Survival of CHO Cells Exposed to Different Concentrations of p-brombenzylisothiocyanate

Concentration of PBBI (µg/ ml medium)	Number of Clones Replicate 1	Number of Clones Replicate 2	Average Number of Clones	% Survival
0.0	531	482	507	100.0
2.5	341	411	380	74.9
5.0	286	294	290	57.2
7.5	97	117	107	21.0
10.0	33	25	29	5.7
12.5	5	1	3	0.6

Table X. Influence of Different Concentrations of PBBI on the Mitotic Index

Concentration of PBBI (µg/ ml medium)	Number of Mitotic Cells	Number of Interphase Cells	Total Number of Cells Counted	Mitotic Index
0.0	34	1127	1161	29.3
1.2	94	1562	1648	57.0*
2.4	67	1022	1089	61.5*
3.6	3	1212	1215	2.5*

* significant at 0.05 level

Fig. IV. Dose- Survival Curve of CHO Cells Exposed to Different Concentrations of PBBI

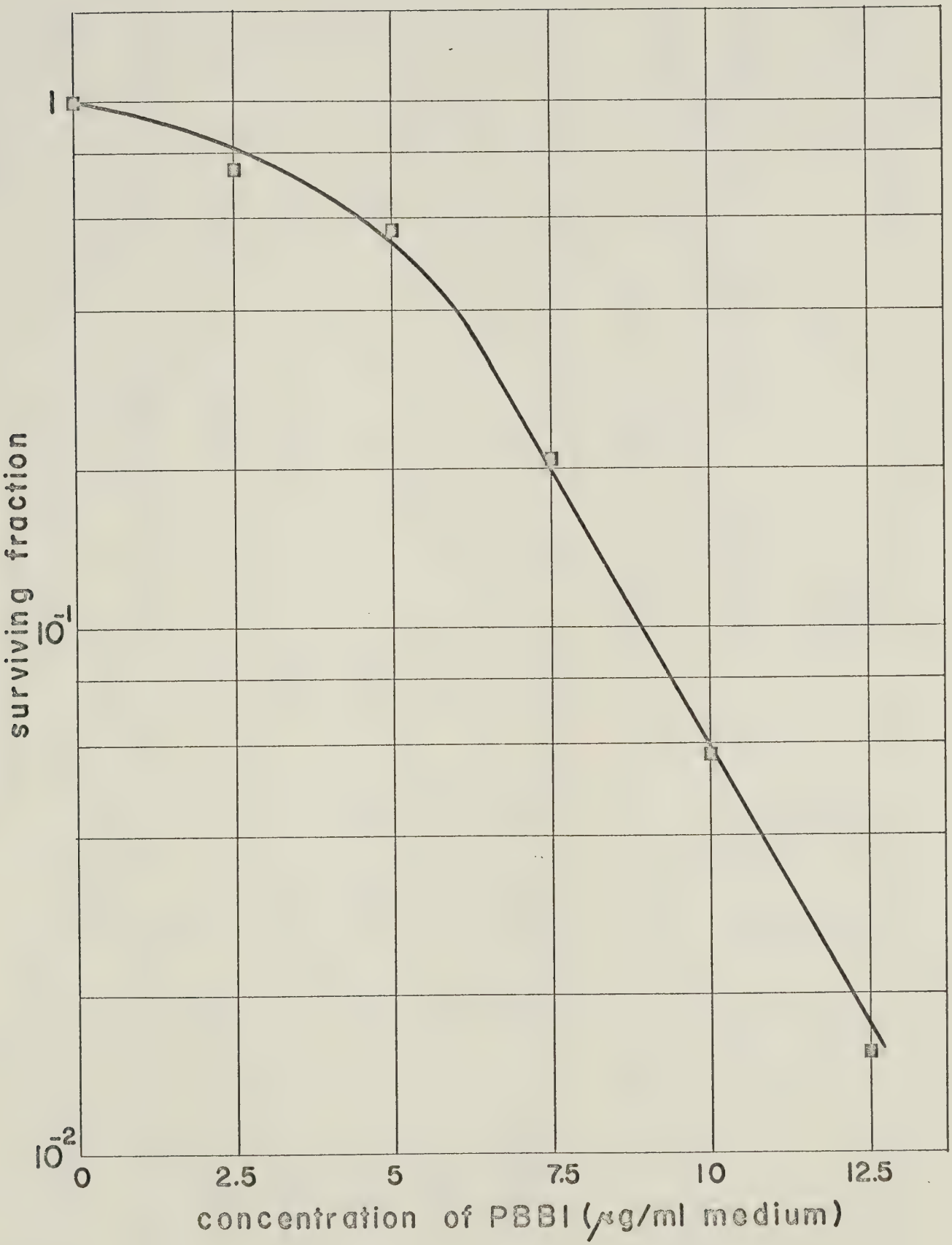

surviving fraction
1
10^{-1}
10^{-2}
0
2.5
5
7.5
10
12.5
concentration of PBBI (μg/ml medium)

Table XI. Effect of PBBI (6 μg/ml) on Chromosomes of CHO Cells at Various Intervals After Treatment

Time (hr)	Treatment	% Metaphases With Chromosome Number						
		20	21	22	polyploid	Chromatid Exchanges	Structural Aberrations Dicentrics	Breaks
4	Control	7.0	89.0	3.0	1.0	-	-	-
	PBBI	4.8	61.3	2.4	29.0	0.8	0.8	-
8	Control	3.0	95.0	1.0	1.0	-	-	-
	PBBI	2.9	61.2	-	33.0	1.0	1.0	-
12	Control	3.0	95.0	2.0	-	-	-	-
	PBBI	6.2	62.5	3.6	26.8	-	0.9	-
24	Control	4.9	94.1	1.0	-	-	-	-
	PBBI	0.9	76.4	1.8	20.0	-	-	0.9
36	Control	2.8	95.3	1.9	-	-	-	-
	PBBI	1.9	77.6	0.9	18.7	0.9	-	-

Table XII. Observations of Chromosomes of Surviving Clones After Exposure to PBBI

Concetration of PBBI (μg/ml)	% Metaphases With Chromosome Number					Structural Aberrations	
	19	20	21	22	polyploid	Dicentrics	Rings
0.0	-	2.0	96.0	1.0	1.0	-	-
2.5	-	3.9	90.1	2.1	3.9	-	-
5.0	1.7	0.9	51.3	-	45.2	0.9	-
7.5	1.0	2.0	55.0	-	42.0	-	-
10.0	0.9	0.9	44.1	0.9	52.9	-	0.9

PBBI was found to have a strong toxic effect on CHO cells. Table IX. and Fig. IV. demonstrate that even very low concentrations of PBBI markedly reduced the surviving fraction of cells. D_{10} was determined to be equal to 8.7 µgPBBI/ml of medium. An interesting effect of PBBI on mitotic activity of CHO cells was noted (Table X). Treatment with 1.2-2.4 µg PBBI/ml for 17 hr resulted in an increased mitotic index, whereas concentrations of 3.6 µg PBBI/ml and higher reduced the mitotic index. In studies of the effects of PBBI on chromosomes, an experimentally convenient dose of 6 µg PBBI/ ml of medium was used. This concentration permits 37% cell survival. As it is evident from Table XI., PBBI treatment greatly increased the number of polyploid cells. An increase in the percentage of polyploid cells is also apparent in clones of CHO cells exposed to PBBI (Table XII.), although an interval of 6 days had elapsed since treatment. PBBI seems to increase the number of metaphases with chromosomal aberrations, but such an effect persists only until 12 hr after removal of the drug from the medium by washing the cells.

4. p-bromphenylisothiocyanate

Table XIII. Survival of CHO Cells Exposed to Different Concentrations of PBFI

Concentration of PBFI (µg/ml)	Number of Clones Replicate			Average Number of Clones	% Survival
	1	2	3		
0	428	455	439	440.7	100.0
18	430	406	414	426.6	96.8
36	385	378	375	379.3	85.9
54	120	133	139	130.6	29.6
72	113	98	85	98.6	22.4
90	76	34	52	54.0	12.3

Fig. V. Dose-Survival Curve of CHO Cells Exposed to Different Concentrations of PBFI

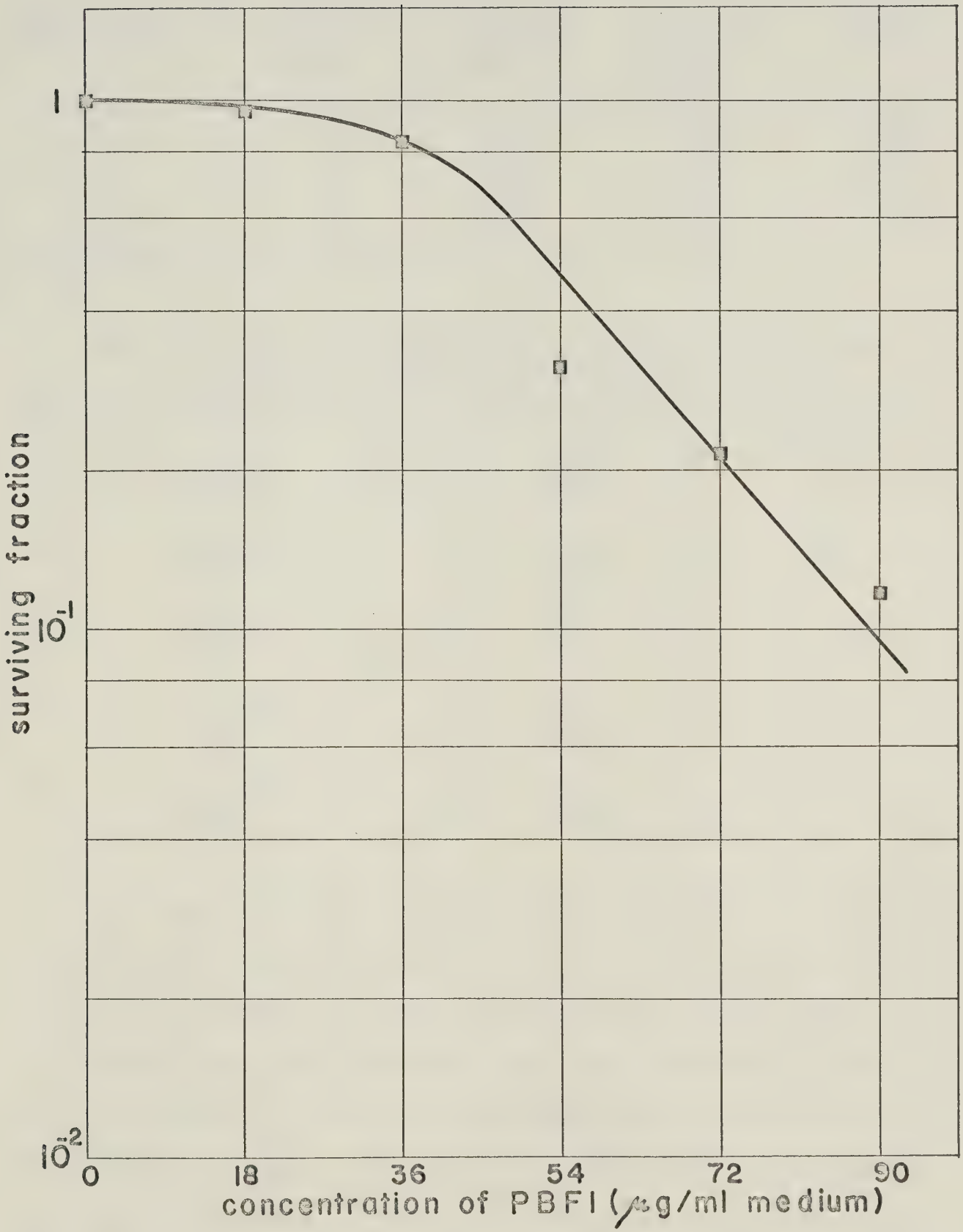

surviving fraction
1
10^{-1}
10^{-2}
0
18
36
54
72
90
concentration of PBFI (μg/ml medium)

Table XIV. Influence of Different Concentrations of PBFI on the Mitotic Index

Concentration of PBFI (µg/ml)	Number of Mitotic Cells	Number of Interphase Cells	Total Number of Cells Counted	Mitotic Index
0	34	1014	1048	33.5
10	17	1251	1268	13.6*
20	15	1268	1283	11.7*
30	14	1628	1642	8.5*
40	11	1210	1222	9.0*

*Significant at the 0.05 level.

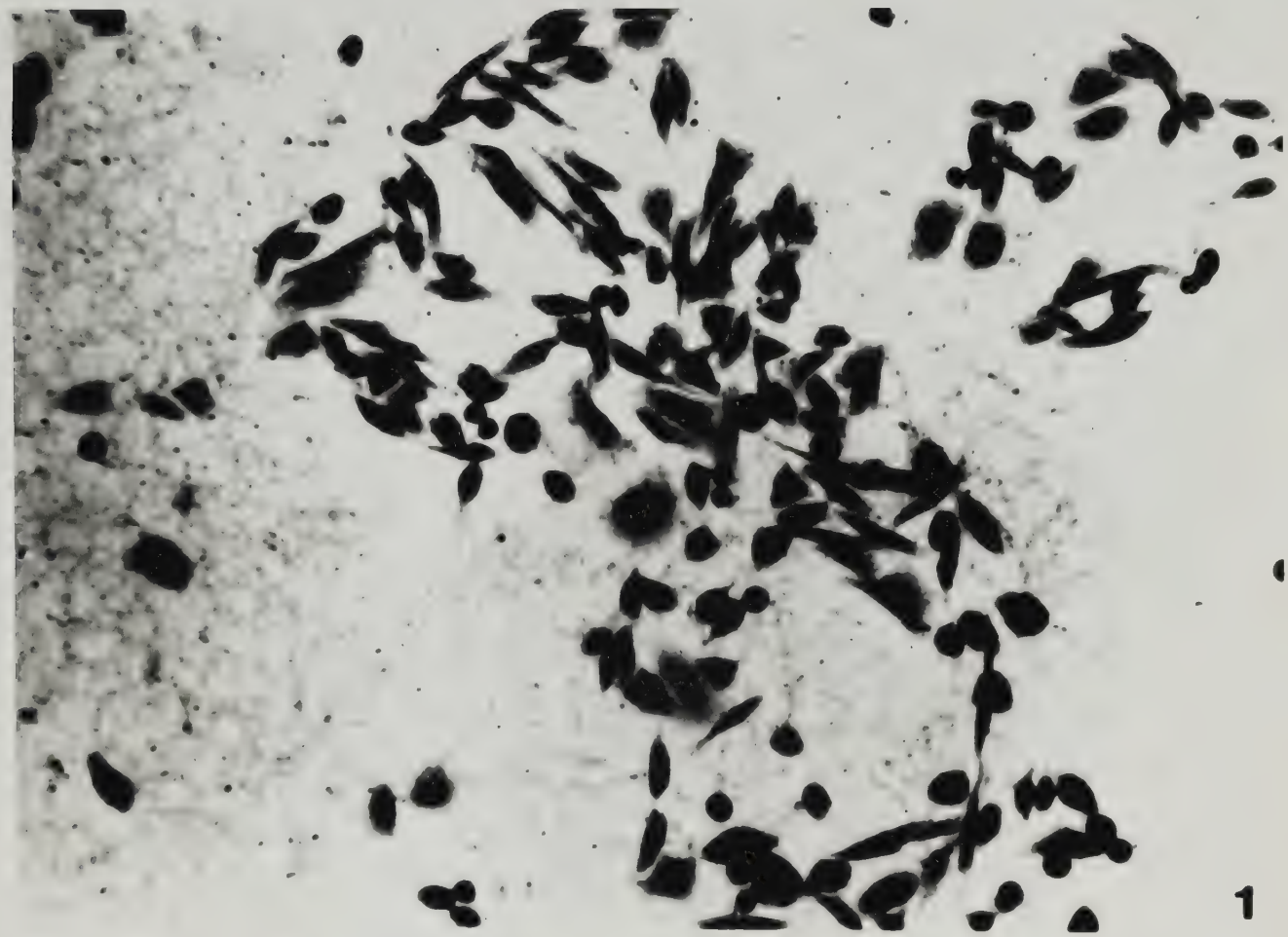
Table XV. Effect of PBFI (50 µg/ml of medium) on Chromosomes of CHO Cells at Various Intervals After Treatment

Time (hr)	Treatment	Percent of Metaphases With Chromosome Number 19	20	21	22	polyploid
4	Control	-	7.0	89.0	3.0	1.0
	PBFI	1.0	2.9	91.1	2.0	3.0
8	Control	-	3.0	95.0	1.0	1.0
	PBFI	1.0	4.8	91.3	1.9	1.0
12	Control	-	2.9	93.1	2.0	2.0
	PBFI	1.9	1.0	94.2	2.9	-
24	Control	-	4.9	94.1	1.0	-
	PBFI	-	5.8	92.2	2.0	-
36	Control	0.9	1.9	95.3	1.9	-
	PBFI	-	1.0	95.0	1.0	-

PBFI exerts relatively fewer toxic effects on CHO cells than PBBI. Table XIII. and Fig. V. demonstrate the survival of CHO cells after treatment with PBFI. The effects of concentrations higher than 90 µg PBFI/ml were not investigated due to the interaction of PBFI with the medium. D_{10} derived from the dose-survival curve amounts to 99 µg PBFI/ml of medium. The presence of PBFI in the medium causes a decrease of the mitotic index, as is evident from Table XIV, and no chromosomal changes were observed when the drug was used in a concentration of 50 µg/ml of medium (Table XV.).

Plate I. Monolayer coverslip culture of Chinese Hamster ovary cellls grown in Leighton Tubes. No drug treatment. (x 4950)

Plate II. Similar CHO culture after treatment with chlorpromazine (24 µg/ml) for 17 hr. Note smaller, more rounded cells. (x 4950)

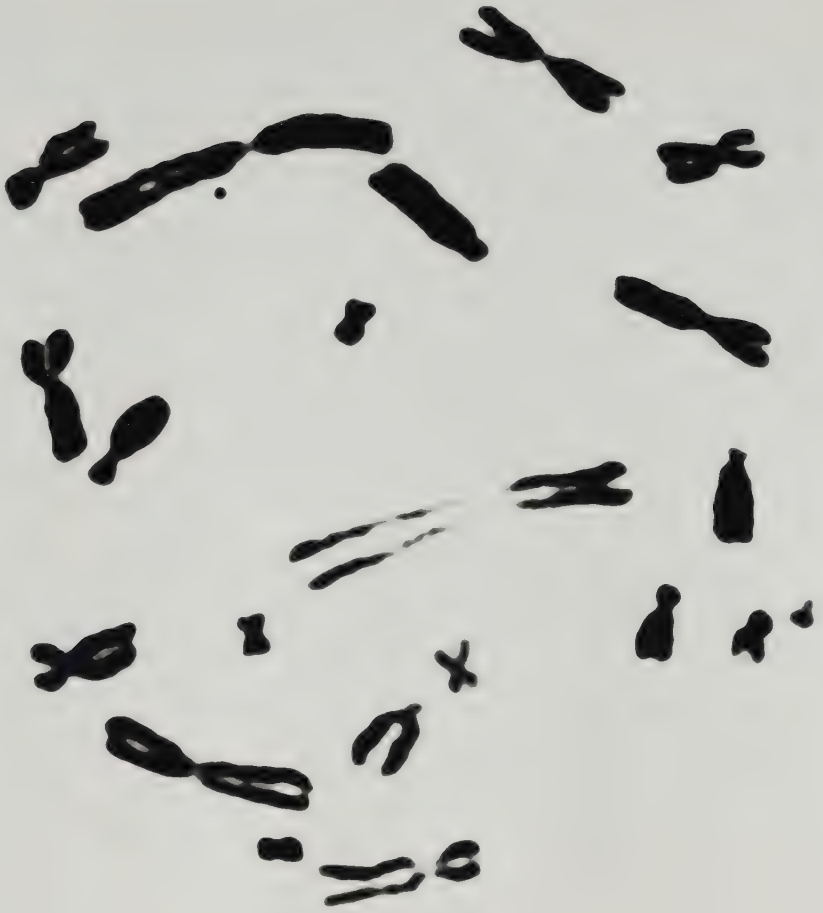


1

2

Plate III. Typical metaphase spread of a CHO cell with 21 chromosomes. (x 7200)

Plate IV. Typical metaphase spread of a CHO cell with 21 chromosomes. (x 7650)



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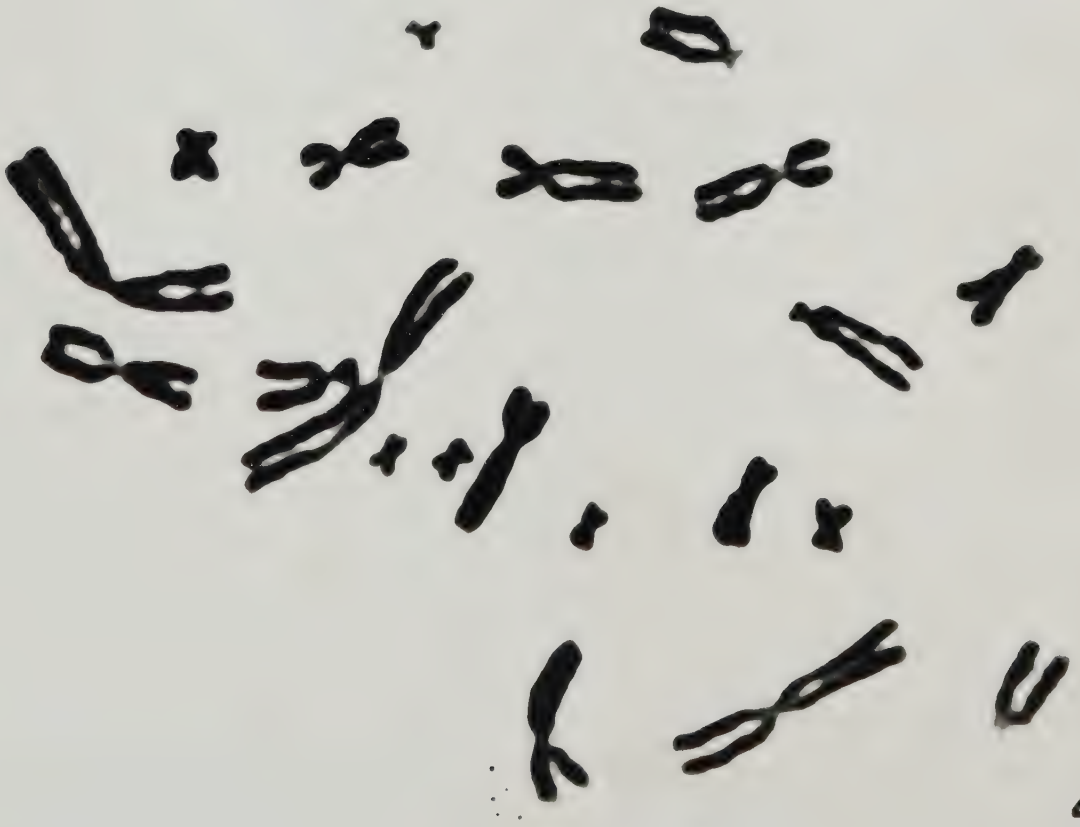
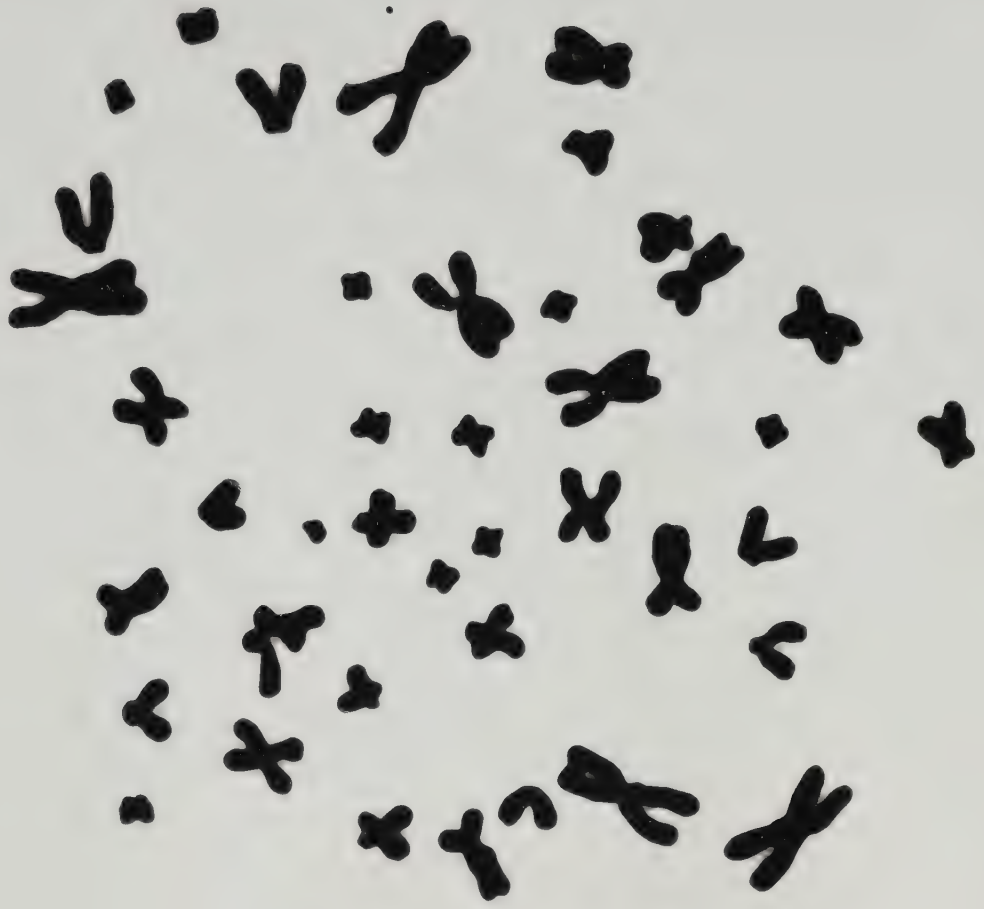


4

Plate V. Polyploid CHO cell. (x 6300)

Plate VI. Polyploid CHO cell. (x 7650)



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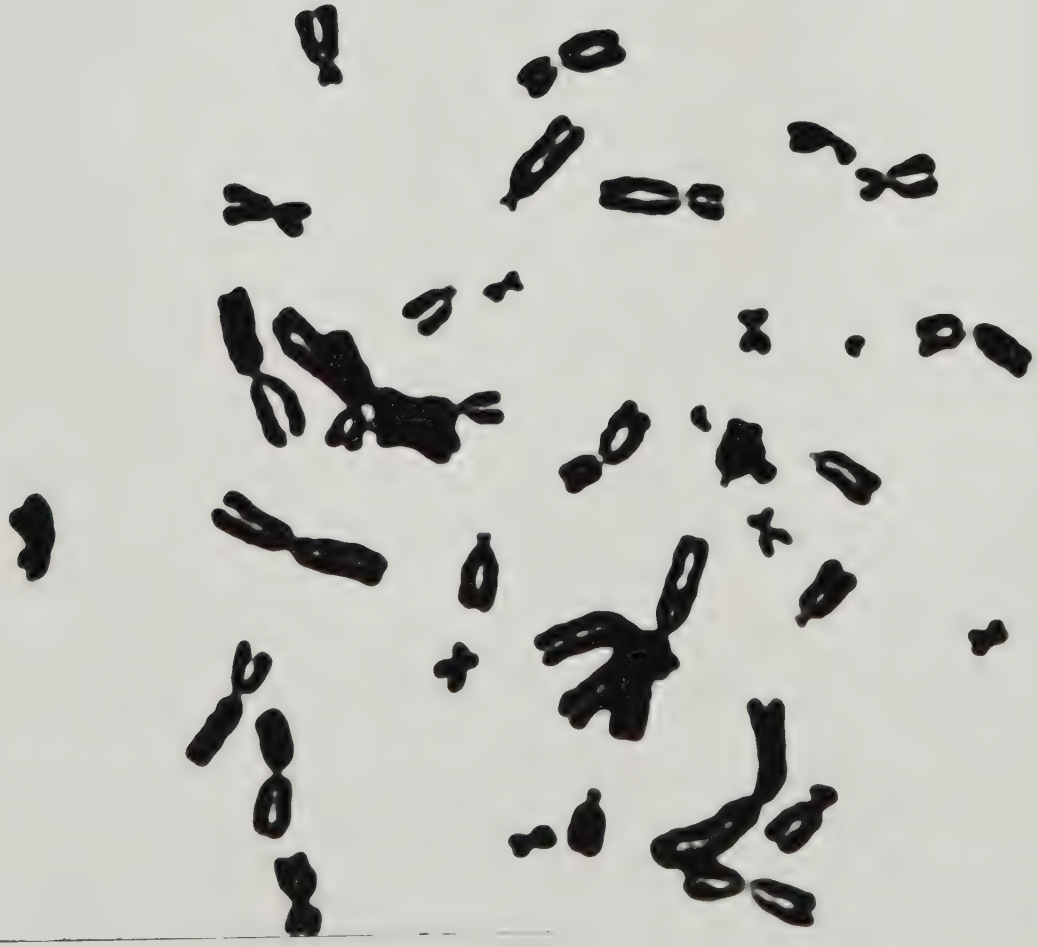
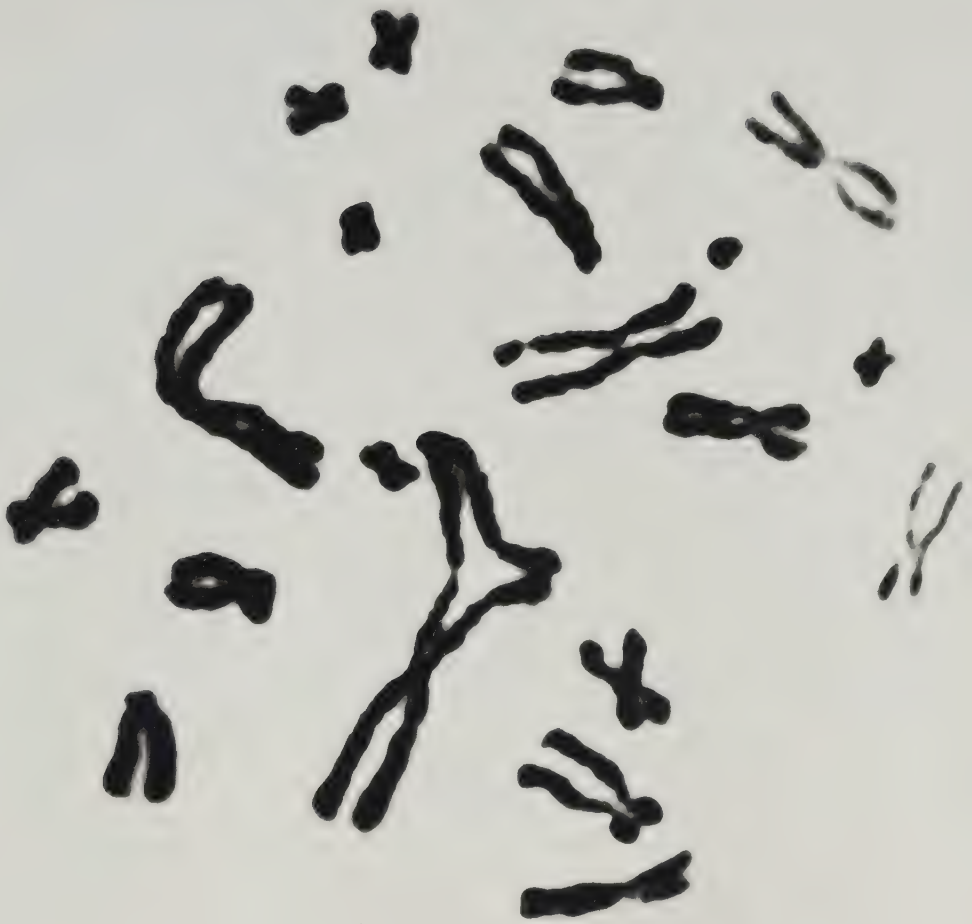


6

Plate VII. CHO cell with chromatid exchange after drug treatment. (x 5400)

Plate VIII. CHO cell with chromatid exchange after drug treatment. (x 5850)



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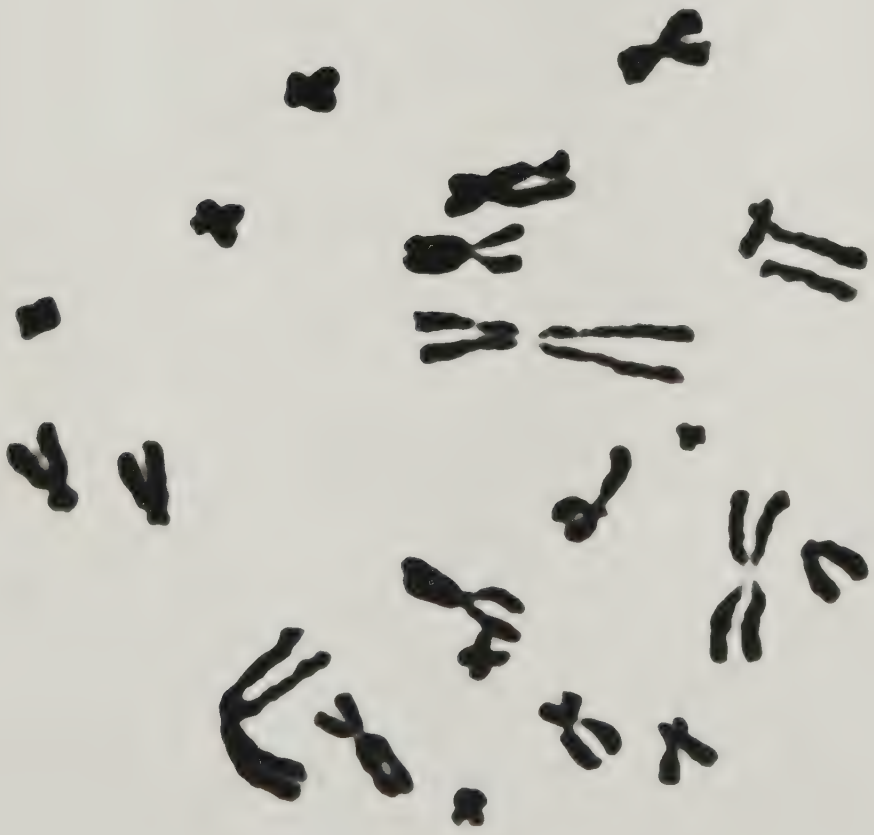

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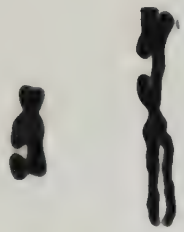
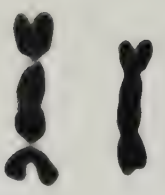
Plate IX. (A.) Chromatid gaps.
(B.) Dicentric chromosomes.
(C.) Ring chromosome.
(D.) Chromatid exchange.
(E.) Chromatid exchange.
(F.) Multiple chromatid exchange.
(G.) Multiple chromatid exchange.
(H.) Chromatid exchange.
(I.) Chromatid exchange and chromatid breaks.

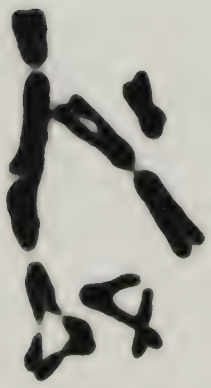


A



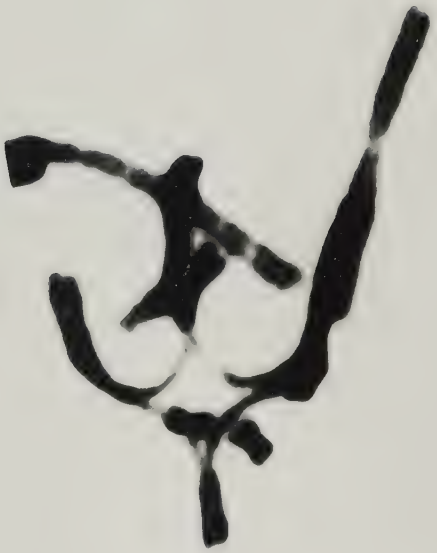
B

C

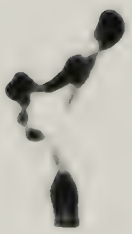


D

E



F



G

H

I

9

DISCUSSION

The present study deals with the effects of four drugs on asynchronously growing CHO cells. Several authors have reported variable sensitivity of cells to chemicals during different phases of the cellular life cycle (Lozzio, 1969; Mauro and Madoc, 1970; Wheeler, et al., 1970). For this reason, the cell cultures were exposed to drugs for one generation time, to ensure that all phases of the life cycle of a particular cell underwent treatment. Since it is known that the generation time of a cell population varies with the experimental conditions employed, this parameter was determined. The duration of the generation time was found to be 16.6 hr, which closely agrees with 15.9 hr determined by Miller (1971) under the same experimental conditions, but with a different subline of CHO cells.

From the results presented, it is evident that all drugs tested influence cell division. CPZ, DMSO, and PBFI were found to suppress mitotic activity at concentrations which only slightly inhibited clone-forming ability. The slight effect on the ability of cells to form clones compared with higher effects on the mitotic index seems to be reasonable in that a drug-induced lag in cell division would be most evident immediately, whereas clones were counted 6 days after treatment. This interval will allow most cells to overcome the lag and give rise to colonies.

Experiments demonstrate that increasing concentrations of CPZ, DMSO and PBFI further decreased the mitotic index. It has been postulated in the literature (see LITERATURE REVIEW) that each of

the tested drugs interferes with certain biochemical processes of the cell. The processes most commonly affected (DNA synthesis, oxidative phosphorylation, etc.) are essential to the completion of mitosis (Kihlman, 1966). As the concentrations increase, these effects generally become more severe. A decrease in mitotic activity of cultured human leucocytes after treatment with CPZ has been observed by others (Westring, et al, 1964; Bloom and Tjio, 1964; Cohen, et al., 1969).

Experimental data indicate that certain concentrations of PBBI increased the mitotic index as well as the number of polyploid figures. Increased polyploidy and mitotic index may be related, since PBBI may affect the mitotic apparatus in such a way as to block nuclear and cellular division. Continuing DNA replication of a cell blocked in mitosis would then result in cell polyploidy.

The dose-survival curve seems to be a very sensitive method for ascertaining drug toxicity, since this method is based on the survival of single cells and not on growth characteristics of cell populations as are other methods. CHO cells are especially suited to this method because of their high plating efficiencies. However, a disadvantage of this method is that the experimental conditions can affect the final shape of the curve. In an attempt to minimize the influence of varying experimental conditions, strict attention was paid to ensure constancy throughout the series of tests.

Cytotoxicity of DMSO was measured by several authors using different survival criteria and different types of cells. Results of the present study could be compared with data of Berliner and

Ruhmann (1967), who tested the influence of DMSO on the growth of fibroblast L-929 cells and observed suppression of growth with 3% DMSO, and no effect on growth with 1% DMSO. In this study, 1% DMSO corresponds to 87% survival; 2% DMSO to 67% survival, and 3.5% of the drug to 7% survival. Chang and Simon (1968) reported the inhibition of growth of HeLa cells with 2% DMSO, and of L-cells with 3% DMSO. Also the results concerning toxicity of isothiocyanates agree to some extent with those from the literature, even when different methods were employed. Horakova (1969) determined the ED_{100} (100% inhibition of growth) value for the action of PBBI (1-5 μg/ml) and PBFI (120 μg/ml) on HeLa cells. Results in this study demonstrate a similar relationship between the toxicity of these two isothiocyanates; approximately ten times greater toxicity for PBBI than PBFI. Small differences in values could be attributed to differences in methods and to different sensitivity of HeLa and CHO cells.

The shapes of dose-survival curves for asynchronous populations of CHO cells exposed to CPZ, DMSO, PBBI and PBFI are similar to those characteristic of many other chemicals such as nitrogen mustard, hydroxylamine, nogalamycin, cycloheximide, caffeine, ethylmethanesulfonate and acridine mustard (Mauro, 1968; Puck, 1969). After the initial shoulder, the curves continue exponentially downward. As was pointed out by Kao and Puck, the

presence of the initial shoulder may indicate the existence of,

> a, a need for a minimum concentration of the agent in the medium in order to overcome processes, e.g. active transport, which prevent the agent from reaching the critical sites within the cell; b, multiplestructures in the cell which must be individually inactivated by the agent employed, or c, cellular repair mechanisms whose capability must first be saturated before damage can be elicited.
>
> (Kao and Puck, 1969, p. 255.)

The last factor seems to be especially important, since the existence of repair of sublethal damage was reported with several chemicals such as sulfur mustard, ethylmethanesulfonate, methylmethanesulfonate, etc. (Hahn, et al., 1968; Mauro and Elkind, 1968). The survival curve for PBFI is not unambiguous. Due to the interaction of certain concentrations of PBFI with phosphates in the medium and a limited supply of this agent, it was not possible to repeat this experiment often enough to obtain clear results.

Although the original goal was to examine the effects of drug concentrations which permitted exactly 30% cell survival, it was found that the desired concentration fell within the exponential fraction of the slope of the survival curve, where a small change in drug concentration resulted in a large change in cell survival. For this reason, the effects of drugs on chromosomes were studied within concentrations at which cell survival ranged from 20-40%.

It is well known that several factors (differences in media, use of antibiotics, methods of distilling water, ways of

maintaining cell cultures, etc.) may influence the frequency of chromosomal changes. In order to negate the possible effects of physical and environmental conditions, experimental procedures were kept as constant as possible, and parallel controls were employed.

The present study of the effects of CPZ on chromosomes of CHO cells in vitro gave negative results, which confirms the work of Cohen, et al. (1969) and Kamada, et al. (1971). These investigators were unable to detect chromosomal damage caused by CPZ in human leucocytes when cultured in vitro. This finding concurs with the theory of Allison and Paton (1965) concerning induction of chromosomal aberrations as a result of damage to lysosomes. CPZ acts as a lysosomal stabilizer and therefore chromosomal damage would not be expected to be caused by this agent.

The observation of chromosomes of CHO cells treated with 0.03 ml DMSO/ml of medium illustrated the importance of chromosome investigation during the first cell generation after treatment. Only during this period could chromosomal aberrations be observed, indicating that cells with chromosomal damage are either eliminated from the culture, or that the damage is repaired during the second cell generation after treatment. Aberrations of the exchange type which resulted in translocations during the following generation cannot readily be recognized in CHO cells, since the karyotype includes chromosomes of a morphology markedly altered from the normal Chinese Hamster complement. Exchange figures, however, can be readily observed during the first cell generation after drug treatment. It is evident from the results obtained that 3% DMSO gives rise to 5-14% aberrations; such a frequency seems to be high

enough to limit the use of this agent to no more than a concentration of approximately 3% in procedures such as storage of frozen cells. It has to be pointed out also that the above cited frequency is based on mitotic cells only and that the actual frequency of aberrations present in the total cell population might be considerably higher.

From literature references, the inhibition of protein synthesis by DMSO seems most likely to be responsible for the induction of chromosomal aberrations. The requirement of de novo protein synthesis for chromosome replication has been reported, however, many questions remain open. It is not known whether the effect of DMSO is direct; ie., on histone metabolism, or indirect; by inhibition of DNA synthesis (Mueller, 1962) or of repair processes. The fact that treatment with a low concentration of DMSO (0.01 ml DMSO/ml of medium) resulted in the absence of chromosomal changes suggests that a threshold concentration of this drug is needed within the cell to produce a chromosomal damaging effect. This confirms the results of Stenchever, et al. (1967) indicating the absence of damage after treatment with 1% DMSO.

The drug PBBI (6 µg/ml of medium was found to cause a marked increase in the number of polyploid cells. This effect seems to be caused by a disturbance of the mitotic apparatus. Supporting evidence for this hypothesis lies in the observation of an increased mitotic index in PBBI-treated cells, indicating a blockage of an essential stage in nuclear and cellular division with concommitant accumulation of cells in metaphase. Other possible explanations include the possibility that polyploid cells enjoy higher selective

advantage over the original (pseudodiploid) karyotype when PBBI is present.

It is surprising that related agents with almost similar chemical structure such as PBBI and PBFI (the difference between these two compounds is a single $-CH_2$ group present in PBBI) have strikingly different biological activities. Toxicity, for example is 10 times greater with PBBI than with PBFI. Moreover, PBFI does not seem to induce chromosomal changes at the concentrations employed. As stated before, the chemical interaction of PBFI with the medium did not permit the effects of higher drug concentrations to be established.

The use of drugs has increased sharply over the last fifty years and although strict legislation governs the introduction of new drugs, a further refinement in the procedures to test the possible harmful side-effects of new and existing drugs remains of paramount importance. For instance chromosome damage, as a result of drug toxicity, can have serious consequences. Damage to cells of the reproductive organs may in turn lead to miscarriages, to birth of individuals carrying undesirable gene mutations, to malformations or to post-natal death. In addition, certain chromosomal rearrangements, such as balanced translocations, may produce an effect which can be only recognized in subsequent generations.

The fact that many of the present drugs are intended for human use, demands that any test for their potential danger to human chromosomes, has to be carried out by means of <u>in vitro</u> studies

using human cell lines or by determining their effect on living animals. Animals require expensive maintenance, and pretentious experimental methods usually lead to end results which are only partly applicable to the human organism, if at all. On the other hand, experiments *in vitro* have the disadvantage that the inherent differences in conditions between between *in vitro* and *in vivo* studies are often unknown and hence the results obtained from *in vitro* studies may not be relevant to living systems. Several reasons may account for these differences: the *in vitro* system makes use of artificial media which are foreign to the *in vivo* system. The response of the cell to a particular drug might be different in the two systems for the simple reason that the drug may be activated or inactivated when applied to an *in vivo* system, whereas the reverse may be true in an *in vitro* cell culture. In addition, the effect of drug transport as it takes place in the living oragnism may have an effect on the action concentration. That is to say, it may affect the final concentration which exerts an organ-specific effect. Therefore, there is no doubt that results obtained from *in vitro* studies have to be interpreted with extreme care, regardless of the fact that cell tissue culture methods constitute a simple technique for rapid screening of drugs.

The availability of different cell types for *in vitro* studies facilitates the choice of a cell system most suitable for the drug under investigation. For instance, in the present study, the selection of a cell line with a relatively low chromosome number, short generation time, high plating efficiency and loose cell attachment to glass during mitosis, was used to full advantage. Since, however,

the drugs tested are of dermatological interest, the evaluation of these drugs should also be based on chromosome studies employing human fibroblast cell lines or strains. Zuckner, et al. (1967) investigated the influence of the drug DMSO on leucocytes originating from patients treated with this drug. The negative results were based on the examination of only 12-17 metaphase plates per patient, and hence, are far from convincing. Because of the increasing interest in the drug DMSO, and especially in view of reported teratogenic side-effects, a comprehensive study of this drug and its biological action should be carried out. The results presented here seem to justify a cautious use of high concentrations of DMSO in histological freezing procedures for protective purposes, since its application may result in a drug-induced change of the genome.

SUMMARY

The effects of CPZ, DMSO, PBBI and PBFI on asynchronously growing CHO cells *in vitro* were studied. It was demonstrated that all tested drugs influence, at the appropriate concentrations, the mitotic activity of cells. CPZ, DMSO and PBFI were found to reduce the mitotic index; PBBI (1.2-2.4 μg/ml of medium) was found to increase the mitotic index. Dose-survival curves for all drugs tested have the same characteristics. After the initial shoulder, the curves continue exponentially. From these curves, the doses of each drug which reduce the survival fraction to 20-40% were obtained and their effects on chromosomes were investigated. Chromosomal aberrations were observed after treatment with DMSO in 5-14% of the metaphases harvested 0-12 hr after the treatment. Exposure of cells to PBBI resulted in an increase of the number of polyploid cells. CPZ and PBFI were not found to cause chromosomal changes.

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